Reversal of Object Recognition Memory Deficit in Perirhinal Cortex-Lesioned Rats and Primates and in Rodent Models of Aging and Alzheimer's Diseases

Mariam Masmudi-Martín [a b †], Irene Navarro-Lobato [a b †], Manuel F. López-Aranda [a b † ‡], Philip G.F. Browning [c ¶], Ana-María Simón [d], Juan F. López-Téllez [a b], Inmaculada Jiménez-Recuerda [a b], Elisa Martín-Montañez [e f], Alberto Pérez-Mediavilla [d], Diana Frechilla [d], Mark G. Baxter [c], Zafar U. Khan [a b f g]

[a] Laboratory of Neurobiology, CIMES, University of Malaga, Campus Teatinos s/n, 29071 Malaga, Spain
[b] Department of Medicine, Faculty of Medicine, University of Malaga, Campus Teatinos s/n, 29071 Malaga, Spain
[c] Department of Neuroscience, Icahn School of Medicine at Mount Sinai, One Gustave L. Levy Place, Box 1065, New York, NY 10029, United States
[d] Division of Neurosciences, CIMA, University of Navarra, Av. Pio XII 55, 31008 Pamplona, Spain
[e] Department of Pharmacology, Faculty of Medicine, University of Malaga, Campus Teatinos s/n, Malaga, Spain
[f] IBIMA, University of Malaga, 29071 Malaga, Spain
[g] CIBERNED, Institute of Health Carlos III, Madrid, Spain
Corresponding author: Zafar U. Khan, zkhan@uma.es

Abstract

The integrity of the perirhinal cortex (PRh) is essential for object recognition memory (ORM) function, and damage to this brain area in animals and humans induces irreversible ORM deficits. Here, we show that activation of area V2, a brain area interconnected with brain circuits of ventral stream and medial temporal lobe that sustain ORM, by expression of regulator of G-protein signaling 14 of 414 amino acids ($RGS14_{414}$) restored ORM in memory-deficient PRh-lesioned rats and nonhuman primates. Furthermore, this treatment was sufficient for full recovery of ORM in rodent models of aging and Alzheimer's disease, conditions thought to affect multiple brain areas. Thus, $RGS14_{414}$-mediated activation of area V2 has therapeutic relevance in the recovery of recognition memory, a type of memory that is primarily affected in patients or individuals with symptoms of memory dysfunction. These findings suggest that area V2 modulates the processing of memory-related information through activation of interconnected brain circuits formed by the participation of distinct brain areas.

Abbreviations

PRh, perirhinal cortex; ORM, object recognition memory; $RGS14_{414,}$ regulator of G-protein signaling 14 of 414 amino acids

Key words
lesions in perirhinal cortex; memory deficits; regulator of G protein signaling; brain memory circuit activation; recovery of memory dysfunctions

Introduction

The perirhinal cortex (PRh) plays a central role in the ability to recognize objects, and it is widely accepted that this brain area makes an essential contribution to object recognition memory (ORM) (Suzuki, 1996, Brown and Aggleton, 2001, Murray and Richmond, 2001, Winters et al., 2008). Ablation of as well as lesions in the PRh cause dramatic deficits in the ability of rats (Mumby and Pinel, 1994, Ennaceur and Aggleton, 1997, Norman and Eacott, 2004), monkeys (Meunier et al., 1993, Buckley and Gaffan, 1998, Buffalo et al., 2000, Hadfield et al., 2003), and humans (Buffalo et al., 1998) to perform ORM tasks. The results of lesion studies suggest that these behavioral impairments result from cell loss in the PRh rather than to the disruption of nerve fibers passing through this region (Baxter and Murray, 2001). The crucial role of PRh neurons in ORM was further confirmed by receptor antagonist infusions in rats (Balderas et al., 2013, Bartko et al., 2014) and monkeys (Tang et al., 1997) and by electrophysiological recordings in neuronal populations that appear to encode recognition memory in rats (Wan et al., 1999, Burke et al., 2012) and primates (Fahy et al., 1993, Hölscher et al., 2003). ORM deficiency arising subsequent to PRh damage is long lasting (Suzuki et al., 1993) and is thought to be irrecoverable.

The regulator of G-protein signaling 14 of 414 amino acids ($RGS14_{414}$) gene is a 1245 base pair splice variant (GenBank, AY987041) cloned from the human cortex. This gene encodes a protein containing 414 amino acids (UniProt, O43566-5), which is expressed throughout the brain (López-Aranda et al., 2006). In contrast to the complete human (GenBank, NP_006471.2) and rat (GenBank, NC_005116.4) genes, $RGS14_{414}$ has a deletion of 153 amino acids within the RGS domain in the N-terminus. This deletion abolishes GTPase activity, a process that is mediated through the RGS domain. Although $RGS14_{414}$ lacks a functional RGS domain, it contains numerous other domains, including a G protein regulatory motif (also called GoLoco) and two Raf-like Ras binding domains (Siderovski et al., 1999, Kimple et al., 2001). Therefore, $RGS14_{414}$ might be involved in other brain functions beyond those requiring GTP hydrolysis. The GTPase activity of the RGS domain is thought to mediate the suppression of intracellular signaling. Accordingly, studies have shown that the complete RGS14 gene (containing the RGS domain) functions as a suppressor of neuronal plasticity and hippocampus-based learning and memory (Lee et al., 2010, Evans et al., 2018). Thus, omission of the RGS domain (as in $RGS14_{414}$) might eliminate this suppressive effect and further aid in memory formation. The facilitation of hippocampal memory processing after the deletion of complete RGS14 from hippocampal CA2 neurons (Lee et al., 2010) further supports this concept. Furthermore, along the same lines, we previously showed that overexpression of $RGS14_{414}$ in area V2 of the visual cortex induces ORM enhancement and that selective elimination of layer 6 neurons in this brain area completely abolished the memory-

enhancing effect of $RGS14_{414}$ (López-Aranda et al., 2009). These findings suggest that layer 6 neurons in area V2 are interconnected with ventral stream circuits that run from the visual cortex to regions of the medial temporal lobe, including the hippocampus. Furthermore, we found that the memory-enhancing effect of $RGS14_{414}$ was associated with BDNF-mediated upregulation of structural plasticity and synaptic remodeling (Masmudi-Martín et al., 2019). Therefore, in this study, we used $RGS14_{414}$-mediated activation of area V2 as a tool to explore whether $RGS14_{414}$ overexpression in area V2 can facilitate memory recovery in PRh-lesioned animals (rats and monkeys), a model in which an area of the brain is selectively damaged, and in models of normal aging and Alzheimer's disease, which are conditions thought to affect multiple brain areas. Our results demonstrate that $RGS14_{414}$ treatment in area V2 was sufficient for the full recovery of ORM in PRh-lesioned animals and in rodent models of aging and Alzheimer's disease.

General methods

Lentivirus preparation

The cDNA of human RGS14 (GenBank accession number AY987041) was cloned into the pLenti6/Ubc/V5-DEST Gateway vector, and RGS14 lentivirus was produced according to the protocols of the ViraPower Lentiviral Expression System (Thermo Fisher Scientific). Vehicle lentivirus (vehicle) was prepared by vector alone.

Animals. Rodents

In this study, we used rats and transgenic mice of Alzheimer's disease. All procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Malaga. Both rats and mice were housed in a temperature-regulated (20 ± 2 °C) room on a 12 h light/dark cycle. Drinking water and food were available *ad libitum* except when indicated. The animals were acclimatized to the room for at least one week before starting the experiments, which took place during the light phase.

Rats. Wistar Han rats were used for this study, and they were obtained from Charles River. Rats of 2–3 months of age were used for study in Fig. 1, Fig. 2; and 3 months (young) and 18 months (aged) of age were used for Fig. 4A. One to two rats were housed in each cage. A total of 181 rats was used in this study.

Transgenic mice with Alzheimer's disease (AD mice). The transgenic hAPPSwInd (J20) mice were from the Jackson Laboratory (stock number 006293). These mice express a mutant form of human APP bearing both Swedish (K670N/M671L) and Indiana (V717F) mutations associated with familial Alzheimer's disease (Mucke et al., 2000). Mutated APP expression is directed to neurons under the control of the human platelet-derived growth factor β chain (PDGFB) promoter. These mice were inbred on the C57BL/6J genetic background. The appearance of Aβ deposition has been observed at the age of 6 months. These mice show synaptic and cognitive impairments, among other characteristics of Alzheimer's disease, and have been widely used for cognitive studies (Palop et al., 2003, Chin et al., 2005). AD mice as well as wild-type C57BL/6J mice of

4 months of age were used for study in Fig. 4B. Four to five mice were housed in each cage. A total of 49 mice was used in this study.

Animals. Rhesus monkeys

Two experimentally naïve male rhesus monkeys (*Macaca mulatta*) were used. Both monkeys were 3 years old at the start of training. The monkeys were housed in social groups in large enclosures with water provided *ad libitum*. The protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of The Icahn School of Medicine at Mount Sinai. Study in monkeys is presented in Fig. 3. In this study, two images (1 and 2) from the beginning and two images (19 and 20) from the end of sequence of images that were used during the test, were included in the analysis.

Statistics

The results were plotted and statistical significance was evaluated using Prism 8. The data used to construct Fig. 1, Fig. 2, Fig. 3, Fig. 4 passed the normality test done by the Shapiro–Wilk normality test, and they showed p values ranged from 0.208 to 0.994. The equality of group variances was tested by the Brown–Forsythe test, and the *p* values ranged from 0.118 to 0.983. Two-group comparisons were analyzed using two-tailed unpaired *t* tests. Multiple group comparisons with single variables were analyzed using one-way ANOVA with Tukey's post hoc test. For multiple group comparisons with more than one variable, two-way ANOVA with the Sidak correction or Tukey's post hoc test was used. A mixed-effects statistical model was used. All data in the figures are presented as the mean ± SEM values.

Experiment 1: RGS14$_{414}$ protein expression in area V2 of rat brain induces ORM enhancement and rescues ORM deficit induced by PRh lesions in rats

Experiment 1: Methods

Lentivirus delivery

Rats were anesthetized with the sevoflurane system (induction at 5% sevoflurane + 1 l/min O_2 and maintenance at 2% sevoflurane + 0.4 l/min O_2) and placed in a stereotaxic frame according to the coordinates obtained from Steriotaxic Coordinates of the Rat Brain by Paxinos and Watson (fourth edition) (Paxinos and Watson, 1998). The coordinates of the injection site into area V2 of visual cortex were AP −4.3, ML ± 2.1, DV −1.9 mm from the bregma. A total volume of 2 µl of RGS14$_{414}$ lentivirus from a stock titer of 2.1×10^7 TU/ml was injected bilaterally through a 30-gauge stainless steel internal cannula at the rate of 8 µl/h. Behavioral tests were performed 3 weeks after injection.

Objects for ORM test

Objects were bottles or containers of different shapes made of plastic or glass. The objects were approximately similar in size to the rats such that the rats were unable to sit on or topple them. The objects were selected according to two criteria: (a) the rats did not show a preference between novel and familiar objects in preference test experiments, and

(b) after exposure to the objects for 3 min, the rats were unable to recall them after a delay of 24 h.

ORM test in rats

The ORM test was performed as described previously (Ennaceur and Delacour, 1988, López-Aranda et al., 2009). Prior to the test, the rats were handled for 8 min daily for five consecutive days, and the next two days, they were habituated in an open field (100 × 100 × 50 cm) for 12 min. On the day of the test and in the object exposure session, the rats were placed in the same open field with two identical objects and were allowed to explore freely for 3 min. After delays of 15, 30, 45 min or 24 h, the animals were tested for ORM status with one previously presented object (familiar) and a novel object. The location of the novel object was changed randomly between the left and right sides. The open field as well as objects were cleaned after each session. Objects shown previously were never presented to the same animal in any sessions. Sessions were video recorded. One test per day was performed. The exploration time from video was calculated by two independent persons without knowledge of the animal conditions. Usually, the variations between observers were 0–2 s. The exploration time was counted only when the animal was touching the object with its nose. Standing using the object as support was not counted. For vehicle- and $RGS14_{414}$ lentivirus-treated animals, the average total exploration times of both identical objects during the object exposure session were 26.52 ± 2.87 s and 26.15 ± 2.49 s, and the average total exploration times of both objects (familiar + novel) during the ORM test session were 28.34 ± 1.97 s and 27.83 ± 2.61 s, respectively.

The discrimination index (DI) presented in Fig. 1, Fig. 2, Fig. 4 was calculated by dividing the time spent exploring the novel object by the total exploration time (familiar object + novel object). A DI equal or less than 0.5 indicated that the animals were unable to retain object information in memory because they explored both familiar and novel objects equal times (familiar object 50% and novel object 50%), whereas a DI above 0.66 was considered to indicate that the animals were able to successfully retain information about the object in memory because animals spent exploring novel object more than 66% of the total time and less than 34% of the time exploring familiar object.

Immunohistochemistry

Immunohistochemistry was performed as described previously (Khan et al., 1998, López-Aranda et al., 2009). In brief, after termination of the behavioral tests, the rats were perfused transcardially with a fixative containing 4% paraformaldehyde, 1.37% l-lysine and 0.21% *meta*-periodate and cryoprotected with 30% sucrose. Coronal brain sections with a thickness of 30 μm were incubated overnight at 4 °C with an affinity-purified rabbit anti-RGS14 antibody (1:30 dilution) that was prepared in our laboratory (López-Aranda et al., 2009). The sections were then incubated for 90 min with Alexa Fluor 568 goat anti-rabbit IgG (1:1000 dilution; A-11011; Thermo Fisher Scientific), and immunofluorescence labeling was detected by confocal microscopy.

Ox7-SAP injection into PRh of rat brain

The injection of Ox7-SAP (0.9 µg in 1 µl; IT-02; Advanced Targeting Systems) into the PRh was performed in a manner similar to that described in the lentivirus delivery section, using the coordinates AP −4.52, ML ± 6.7, DV −4.75 mm from the bregma. The extent of damage in the brains of these animals was analyzed after staining the brain sections with Cresyl Violet.

Cresyl Violet staining

Brain sections of rats mounted on gelatin-coated slides were stained for 15 min with 1% Cresyl Violet. The sections were dehydrated and mounted for analysis under a microscope.

Experiment 1: Results

In this study, a lentivirus of the $RGS14_{414}$ gene was delivered to area V2 of the brains of Wistar Han rats to induce expression of the $RGS14_{414}$ protein. After 3 weeks, the memory status of these animals was evaluated by their performance on the ORM task. When normal untreated rats of 3 months old were exposed to an object for 3 min, they were able to retain information about the object in memory after a delay of 15, 30 or 45 min but not after a delay of 24 h (Fig. 1A; one-way ANOVA, $F(3, 38) = 20.59$; 15, 30 and 45 min versus 24 h, Tukey's multiple comparisons test, $p < 0.0001$). However, rats treated with the $RGS14_{414}$ gene in area V2 were able to retain the same object information in memory after a delay of 24 h (Fig. 1B; one-way ANOVA, $F(3, 40) = 26.51$; RGS14 versus vehicle, Tukey's multiple comparisons test, $p < 0.0001$). Treatment of rats with lentivirus of empty plasmid (vehicle), saline solution or even lentivirus of RGS12, a protein that belongs to the same family as $RGS14_{414}$, did not induce this object information to be retained in memory (Fig. 1B; one-way ANOVA, $F(3, 40) = 26.51$; vehicle versus saline or RGS12, Tukey's multiple comparisons test, $p > 0.99$), and the performance of these rats in the test was similar to that of untreated rats (24 h in Fig. 1A). In addition, treatment of the parietal cortex, another brain area, with the $RGS14_{414}$ gene produced no effect on ORM (Fig. S1). Together, these results suggest that $RGS14_{414}$-mediated ORM enhancement was specific. After termination of the behavioral studies, the brains of the rats were processed to study the affected surface area after $RGS14_{414}$ lentivirus injection. Both images of a coronal brain section after immunostaining with antibodies specific for RGS14 (Fig. 1C) and a depiction of coronal brain sections after analysis of serial sections, showing maximum expansion of the $RGS14_{414}$ protein in area V2 (drawings in red color in Fig. 1D), indicated that $RGS14_{414}$ protein expression was confined to area V2.

To test whether $RGS14_{414}$ treatment can recover ORM in PRh-lesioned rats, memory-deficient rats were generated by induction of lesions to the PRh through an injection of Ox7-SAP, which is a saporin-based immunotoxin that causes selective eradication of neurons and does not affect other brain structures or passing nerve fibers (Nolan and Freeman, 2005, Traissard et al., 2007). PRh-lesioned rats showed considerable ORM deficits compared with untreated normal rats (Fig. 2A; one-way ANOVA, $F(5, 60) = 47.17$; untreated versus PRh lesion, Tukey's multiple comparisons test, $p < 0.0001$). These PRh-lesioned rats were then used for testing whether $RGS14_{414}$ gene treatment in

area V2 could recover ORM. We found that this treatment was sufficient to recuperate the memory in these rats (Fig. 2A; one-way ANOVA, $F(5, 60) = 47.17$; 45 min delay, PRh lesion versus PRh lesion + RGS14 in area V2, Tukey's multiple comparisons test, $p < 0.0001$), and they behaved similar to untreated normal rats (Fig. 2A; untreated). Additionally, we found that $RGS14_{414}$ gene treatment caused further enhancement of ORM in PRh-lesioned rats because, in contrast to untreated normal rats (Fig. 1A), $RGS14_{414}$-treated animals were able to retain object information in memory after a delay of 24 h (Fig. 2A; one-way ANOVA, $F(5, 60) = 47.17$; 24 h delay, PRh lesion + RGS14 in area V2 versus PRh lesion + vehicle in area V2, Tukey's multiple comparisons test, $p < 0.0001$). However, vehicle treatment did not induce any change in ORM at delays of 45 min and 24 h (Fig. 2A; PRh lesion + vehicle in area V2). After termination of the behavioral studies, the brains of the PRh-lesioned rats were processed to study the affected surface area after Ox7-SAP treatment of the PRh (Fig. 2B). Coronal brain sections obtained after analysis of the serial sections are shown to provide a view of the maximum affected area after lesion to the PRh (Fig. 2C). We found that substantial damage remained in the PRh area even after termination of all behavioral studies.

Experiment 2: RGS14414 treatment in foveal area V2 rescues ORM deficit induced by PRh lesions in Rhesus monkeys

Experiment 2: Methods

NMDA injections into the PRh of rhesus monkeys

After stable performance of over 90% on DNMS task (see below), monkeys underwent surgery for the injection of NMDA into the PRh to induce an excitotoxic lesion. Brain surgery was performed in a dedicated operating theater under aseptic conditions. The temporal muscle was detached from the zygoma and retracted. A bone flap was raised and extended ventrally with rongeurs. An orbital approach and a lateral approach were used to administer 20–25 handheld injections of 1 µl of 0.3 M NMDA (0114; Tocris Bioscience) into the PRh of both hemispheres. Six days postsurgery, T2-weighted MRI scans were acquired to determine the extent of the lesion. For the MRI scan, monkeys were placed in a stereotaxic frame, and the precise position of the head within the frame was measured using a tooth marker and manipulator (Kopf Instruments). Visual inspection of scans indicated the location of any spared PRh and its position relative to the ear bars. After 2 weeks of recovery, both monkeys were operated a second time to administer stereotaxically guided injections into the remaining PRh to complete the lesion in both hemisphere. For this second surgery, monkeys were placed in an identical position in the frame as the previous MRI scan with the aid of the tooth marker. In this way, the relative positions of the ear bars and the spared PRh was maintained. MRI scan was done to ensure the success of surgery.

Injections of RGS14 lentivirus into foveal V2 of rhesus monkeys

The location of foveal V2 on the surface of the cortex was estimated according to previous studies (Gattass et al., 1981). During surgery, a small bone flap was created over the

lateral occipital lobe to allow direct visualization of the lunate sulcus and the anterior part of the intraoccipital sulcus. Six 1 μl injections of $RGS14_{414}$ lentivirus from a stock titre of 2.1×10^7 TU/ml, spaced 3 mm apart from one another, were delivered to the foveal V2 in each hemisphere in a 2 × 3 (horizontal × vertical) grid into the cortex just posterior to the ventral tip of the lunate sulcus but not extending into the intraoccipital sulcus. The needle was left in place for several seconds during and after each injection.

Delayed nonmatching-to-sample (DNMS) procedure, training and testing in rhesus monkeys

Training was performed in an automated apparatus consisting of a computer-controlled touch-sensitive monitor (380 mm wide and 280 mm high) on which the stimuli were presented. The stimuli used were clipart objects of 128 × 128 pixels and approximately 60 mm^2 in size. Monkeys sat in a wheeled transport cage 150 mm from the screen and responded to the stimuli by touching the objects. Each touch was registered by the computer, and banana-flavored pellets (190 mg; Noyes Company Inc.) were delivered to a food hopper placed below the monitor after correct responses. A single large food reward was delivered at the end of each session. Once the animals learned to respond to the touchscreen and to retrieve rewards, they were trained on the DNMS procedure.

The DNMS training consisted of 100 trials per session and two consecutive sessions per day. A new set of stimuli was used in each trial. Each trial of the training procedure included a sample phase and a choice phase. In the sample phase, a clipart object was presented in the center of the screen, and the monkey had to touch it to proceed. Touching the object caused it to disappear after 500 ms. After a 2 s delay, in the choice phase, two objects appeared at the left and right positions on the screen where one was the previously shown sample object and the other was a novel object. The monkey was rewarded to touch the novel stimulus. After a correct response, a reward pellet was delivered, and both objects remained on the screen for 500 ms. However, if the response was incorrect, both objects disappeared immediately, and the animal did not receive the reward. After reaching a criterion of 90% correct choices, monkeys were trained on a list of sample objects working from 2 to 20 by gradually increasing the number of sample objects that were presented one after another; the monkey touched each object to proceed to the next object. After going through the list of samples, in the choice phase, the animals were presented with one previously seen object together with a novel object in the reverse order of the list of sample objects. For example, after working through samples, S1, S2, S3……. S20, monkeys would be presented with choices S20 vs N20, S19 vs N19, S18 vs N18.... S1 vs N1, where N# represents unique novel objects. Once the monkeys were capable of working with a list of 20 sample objects, the delay between the sample session and the choice session was increased to 30 s. In this way, the delay between any one sample and its associated choice trial ranged from 30 s (at the list position 20) to approximately 20 min (at the list position 1). Monkeys were then trained to work through 2 and then 3 lists of 20 samples and choices in a single testing session. The final sessions that made up the 15-day performance tests consisted of 60 trial sessions with 3 lists of 20 samples and their respective choices.

Immunohistochemistry

Immunohistochemistry was performed as described above in Experiment 1: Methods section. After termination of the behavioral tests, the monkeys were perfused first with physiological saline and then with 4% formalin and their coronal brain sections were processed for immunohistochemistry.

Cresyl Violet staining

Brain sections of monkeys mounted on gelatin-coated slides were processed for Cresyl Violet staining as described above in Experiment 1: Methods section.

Experiment 2: Results

We next explored whether treatment of area V2 could recover ORM in ORM-deficient rhesus monkeys. We chose to perform this test in nonhuman primates because they have comparable brain structure and circuit complexity to humans. Additionally, results acquired from monkeys would not only confirm the results in rats but also validate the approach of area V2 activation-mediated reversal of ORM deficit. For this study, ORM deficit was first developed in rhesus monkeys (*Macaca mulatta*) by inducing lesions to the PRh (Meunier et al., 1993, Hadfield et al., 2003), and then, the monkeys were subjected to $RGS14_{414}$ gene treatment of area V2 to investigate the reversal in memory deficit. Two male rhesus monkeys of 3 years of age were pretrained to respond to a touchscreen and retrieve rewards and were then trained on the delayed nonmatching-to-sample (DNMS) task. In this test, the animals were shown 20 clipart objects displayed one after another, and the delay between a sample object presentation and its associated choice trial ranged from 30 s (at list position 1) to approximately 20 min (at list position 20). After stable performance for 15 days with $87.78 \pm 2.94\%$ correct choice for the longest delay period of approximately 20 min and list positions 19–20, the monkeys were injected with excitotoxic concentrations of N-methyl-d-aspartate (NMDA) into the PRh of both hemispheres of the brain to induce lesions. We observed that as the number of objects increased, lesioned monkeys exhibited a continuous decline in their performance on the DNMS test and ultimately reached a point where their correct response level was far lower than that during the prelesion stage (Fig. 3A; images 19–20, prelesion versus postlesion, two-tailed unpaired t test, $p < 0.0001$). They showed a relatively mild effect in the choice trials that only required 1–2 objects to be held in memory for 30 s to 2 min (Fig. 3B; two-way ANOVA, $F(2, 9) = 33.5$; images 1–2, prelesion versus postlesion, Tukey's multiple comparisons test, $p = 0.0323$), but their performance was severely degraded and substantial memory deficit was observed in choice trials at list positions 19 and 20 with a delay period of approximately 20 min (Fig. 3B; two-way ANOVA, $F(2, 9) = 33.5$; images 19–20, prelesion versus postlesion, Tukey's multiple comparisons test, $p < 0.0001$). After confirmation of memory deficit, monkeys were treated with the $RGS14_{414}$ gene in area V2 (foveal V2 according to Gattass et al. (1981)) of the visual cortex, and their memory status was evaluated by the DNMS test. The results showed that $RGS14_{414}$ treatment to area V2 was sufficient to recover the memory deficit induced by lesions to the PRh (Fig. 3B; two-way ANOVA, $F(2 ,9) = 33.5$; images 19–20; postlesion versus lesion + RGS14, Tukey's multiple comparisons test, $p < 0.0001$). The level of

ORM after recuperation was similar to that prior to lesion to the PRh in these monkeys. In contrast, memory-deficient monkeys who did not receive $RGS14_{414}$ treatment showed no improvement in memory (Fig. S2). This finding suggests that the recovery observed after $RGS14_{414}$ treatment in monkeys was not due to a possible recovery of function with time. After the behavioral studies, the brains of the monkeys were processed for fluorescence immunohistochemistry using specific antibodies against RGS14 (López-Aranda et al., 2009) to confirm the expression of $RGS14_{414}$ protein in area V2 (Fig. 3C) and were stained with Cresyl Violet to analyze the expansion of lesions in the PRh (Fig. 3D). The damaged area was then plotted from serial sections (Fig. 3E). Although the PRh lesion was not extensively severe, it was sufficient to produce considerable deficits in ORM. Similarly, different grades of damage to the PRh have previously been shown to induce recognition memory deficits in experimental animals (Buckley and Gaffan, 1998, Albasser et al., 2009). Overall, the results from monkeys have shown that the strategy of area V2 activation by the $RGS14_{414}$ gene treatment has specific therapeutic relevance in the reversal of memory deficits in human.

Experiment 3: $RGS14_{414}$ treatment in area V2 rescues ORM deficit in aging rats and Alzheimer's disease mice

Experiment 3: Methods

Lentivirus delivery

Stereotaxic lentivirus delivery in aging rats and AD mice were done similar to as described above in Experiment 1: Methods section using the coordinates obtained from Stereotaxic Coordinates of the Rat Brain by Paxinos and Watson (fourth edition) (Paxinos and Watson, 1998) and The Mouse Brain by Paxinos and Franklin (second edition) (Paxinos and Franklin, 2001). The coordinates of the injection site into area V2 of visual cortex were AP −4.3, ML ± 2.1, DV −1.9 mm from the bregma (rats) and AP −2.3, ML ± 1.3, DV −0.7 mm from the bregma (mice). Behavioral tests were performed 3 weeks after injection.

Objects for ORM test

For study in aging rats, objects were bottles or containers of different shapes made of plastic or glass, whereas for study in AD mice, objects were toys, small bottles and decoration objects made of plastic or glass. The criteria for selection of objects to use in experiments with rats was as described above in Experiment 1: Methods section and the criteria for selection of objects to use in experiments with mice was: a) both AD and wild-type mice did not show preference between novel and familiar objects in preference test experiments, and b) after exposure to objects twice for 10 min each with 10 min apart (Escribano et al., 2009), wild-type mice were able to recall after a delay of 24 h, whereas AD mice were unable to do so.

ORM test in aging rats

The ORM test was performed as described above in Experiment 1: Methods section. The DI presented in Fig. 4A was calculated as above in Experiment 1: Methods section. Prior to the ORM test after 5 months of treatment, rats were again handled and habituated as above in Experiment 1: Methods section and new set of objects that were unseen before by these animals, were used in the test. The average total exploration time of objects (familiar + novel) during ORM test session of vehicle and $RGS14_{414}$ lentivirus treated animals was 23.62 ± 2.37 s and 24.81 ± 2.46 s, respectively.

ORM test in AD mice

The ORM test in AD mice was very similar to that in rats as described above in Experiment 1: Methods section with some exceptions (Escribano et al., 2009). In the object exposure session, the mice were exposed twice for 10 min each with two identical objects 10 min apart. The size of the open field was smaller (50 × 35 × 50 cm). In the ORM test session, the retention of object information in memory was evaluated after a delay of 24 h. The DI presented in Fig. 4B was calculated as above in Experiment 1: Methods section. Prior to the ORM test after 5 months of treatment, AD mice were again handled and habituated as above in Experiment 1: Methods section and new set of objects that were unseen before by these animals, were used in the test. The average total exploration time of objects (familiar + novel) during ORM test session of vehicle and $RGS14_{414}$ lentivirus treated AD mice was 19.75 ± 2.98 s and 20.34 ± 2.76 s, respectively.

Experiment 3: Results

Our PRh lesion studies in both rats and monkeys suggest that ORM deficits are reversible. To some extent, the ORM deficit observed after PRh damage might mimic the ORM deficit observed in neurological and neurodegenerative diseases. Therefore, we next examined whether $RGS14_{414}$ gene treatment in area V2 could reverse memory deficits observed during normal aging and in Alzheimer's disease, the two most representative conditions in which substantial decline in recognition memory has consistently been reported (Walsh and Selkoe, 2004, Robitsek et al., 2008). For this study, we used normal aging Wistar rats and a transgenic *hAPPSwInd* mouse model of Alzheimer's disease (AD mice). We found that untreated young rats at 3 months of age (young untreated) were able to retain information about an object in memory; however, when these rats reached 18 months of age (aged untreated), a noticeable decrease in ORM was observed, with recognition memory falling to a level at which the rats were unable to recall the same information (Fig. 4A; one-way ANOVA, $F(5, 55) = 16.30$; young untreated versus aged untreated, Tukey's multiple comparisons test, $p < 0.0001$). After treating these memory-deficient aged rats with the $RGS14_{414}$ gene, full recovery of ORM was observed (Fig. 4A, one-way ANOVA, $F(5, 55) = 16.30$; 1 month after treatment, aged + RGS14 versus aged untreated, Tukey's multiple comparisons test, $p < 0.0001$), and the performance of the rats reached a level similar to that of young untreated animals. The level of recovered ORM was maintained even after 5 months of treatment (Fig. 4A; one-way ANOVA, $F(5, 55) = 16.30$; 5 months after treatment, aged + RGS14 versus aged untreated, Tukey's multiple comparisons test, $p < 0.0001$). In contrast, vehicle treatment did not induce any effect on recognition memory, and the ORM levels of vehicle-treated rats were similar to

those of untreated aged rats (Fig. 4A; one-way ANOVA, $F(5, 55) = 16.30$; 1 month and 5 months after treatment, aged + vehicle versus aged untreated, Tukey's multiple comparisons test, $p > 0.999$). We could not follow up these $RGS14_{414}$-treated aging rats at later time points because they were unable to perform the ORM test at an advanced age of more than 26 months.

As described for aging rats, the ORM test was used to evaluate recognition memory in AD mice. In the ORM test, AD mice showed a noticeable ORM deficit at the age of 4 months (Fig. 4B; one-way ANOVA, $F(5, 63) = 22.52$; AD versus wild type, Tukey's multiple comparisons test, $p < 0.0001$). Treatment of these memory-deficient AD mice with the $RGS14_{414}$ gene in area V2 led to full recovery of recognition memory (Fig. 4B; one-way ANOVA, $F(5, 63) = 22.52$; 1 month after treatment, AD versus AD + RGS14, Tukey's multiple comparisons test, $p < 0.0001$), and these mice showed ORM levels similar to those of wild-type mice. The level of recovered ORM was maintained even after 5 months of treatment (Fig. 4B; one-way ANOVA, $F(5, 63) = 22.52$; 5 months after treatment, AD versus AD + RGS14, Tukey's multiple comparisons test, $p < 0.0001$). However, vehicle treatment showed no effect on recognition memory, and the ORM levels of vehicle-treated mice were similar to those of untreated AD mice (Fig. 4B; one-way ANOVA, $F(5, 63) = 22.52$; 1 month and 5 months after treatment, AD + vehicle versus AD, Tukey's multiple comparisons test, $p > 0.997$). We further followed on to determine how long the effect of $RGS14_{414}$ treatment can last in AD mice. $RGS14_{414}$-treated AD mice maintained normal ORM levels until the age of 10 months (5 months after treatment); however, at the age of 13 months, these mice failed to sustain this level, which began to decline (Fig. S3). Therefore, the effect of $RGS14_{414}$ treatment on memory in AD mice was long-term, but it was not permanent.

Discussion

Our findings demonstrate that treatment of area V2 with the $RGS14_{414}$ gene is sufficient to restore ORM in PRh-lesioned rats and monkeys and that the treatment outcome is similar in both species even though the lesions were induced by two different methods. The results of these lesion studies suggest that ORM deficits are reversible when the PRh is damaged. We believe that the ORM deficit observed after PRh damage partially mimics the memory deficiency observed during aging and in many neurological and neurodegenerative diseases. Thus, area V2 activation by treatment with the $RGS14_{414}$ gene might have therapeutic relevance in the recovery of recognition memory, a type of memory that is primarily affected in patients or individuals with symptoms of memory dysfunction (Brickman and Stern, 2009). Moreover, caudal visual cortical areas often remain unharmed in patients with neurodegenerative diseases such as Alzheimer's disease; thus, area V2 may be particularly useful for restoration of memory in such conditions. The recovery of ORM in memory-deficient PRh-lesioned animals likely did not occur due to repair of the lesions in the PRh, because damage remained after the termination of behavioral studies; however, intervention of the remaining undamaged PRh neurons in ORM processing cannot be ruled out. Considering that area V2 is interconnected with the entire ventral stream which runs from the primary visual cortex

to regions of the medial temporal lobe, including hippocampus, and is crucial for memory processing (Khan et al., 2011) and that $RGS14_{414}$ gene treatment induces dendritic arborization and synaptic remodeling (Masmudi-Martín et al., 2019), we hypothesize that area V2 might modulate the processing of visual information through activation of these interconnected brain circuits. Memory is thought to be processed through specialized brain circuits formed by the participation of distinct brain areas, and memory deficit is a consequence of reduced activity within these circuits (Hof and Morrison, 2004, Dickerson and Eichenbaum, 2010, Samson and Barnes, 2013). Consistent with this idea, poor performance in patients with memory deficiency has been shown to be associated with decreased functional activity in neural networks in the medial temporal lobe and neocortex (Daselaar et al., 2003, Dickerson and Eichenbaum, 2010). Therefore, it is plausible that RGS14-mediated activation of area V2 restores activity in interconnected ORM-dedicated brain networks in the ventral stream and medial temporal lobe. This idea is consistent with the results of ORM restoration in PRh-lesioned animals, in which only one brain area is damaged, and in animal models of aging and Alzheimer's disease, in which multiple brain areas are thought to be affected. Enhancement of the activity of this ORM circuit could thus explain the reversal in memory deficits in memory-deficient animal models of aging and Alzheimeŕs disease. Therefore, our results emphasize the relevance of interconnected memory circuits in the recovery of ORM. Neurotoxic lesions, as we used in our experiments, do not affect passing nerve fibers (Baxter and Murray, 2001, Nolan and Freeman, 2005, Traissard et al., 2007); hence, neural networks passing through the PRh are expected to be unharmed in lesioned animals, and intercommunications among other brain areas are unlikely to be interrupted. Therefore, damage to the PRh might not affect ORM recovery mediated by $RGS14_{414}$ and area V2, and the activation of brain networks that sustain ORM may be sufficient to override the ORM deficit.

In contrast to the current understanding, our findings demonstrated that ORM deficiency induced by PRh lesions is recoverable. Furthermore, the use of rodent models of aging and Alzheimer's disease extended this idea to show the relevance of area V2 in remediation of memory deficiency and demonstrate that the recovery of ORM relies more heavily on the activation of brain ORM circuits. Additionally, the finding that $RGS14_{414}$ treatment and area V2 may help to restore recognition memory deficits may provide a platform for developing therapeutic strategies for other brain diseases in which neuronal circuits are compromised.

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Author contributions

Z.U.K. developed the overall research concept and the project; M.M., I.N, M.F.L., M.G.B., P.G-F.B. and Z.U.K. designed the experiments; M.M., I.N., M.F.L., P.G.F.B. and A. S. performed the experiments; J.F.L., I.J., E.M., A.P. and D.F. assisted with the experiments; and M.M., I.N., M.F.L., P.G.F.B. and Z.U.K. wrote the manuscript.

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Figures

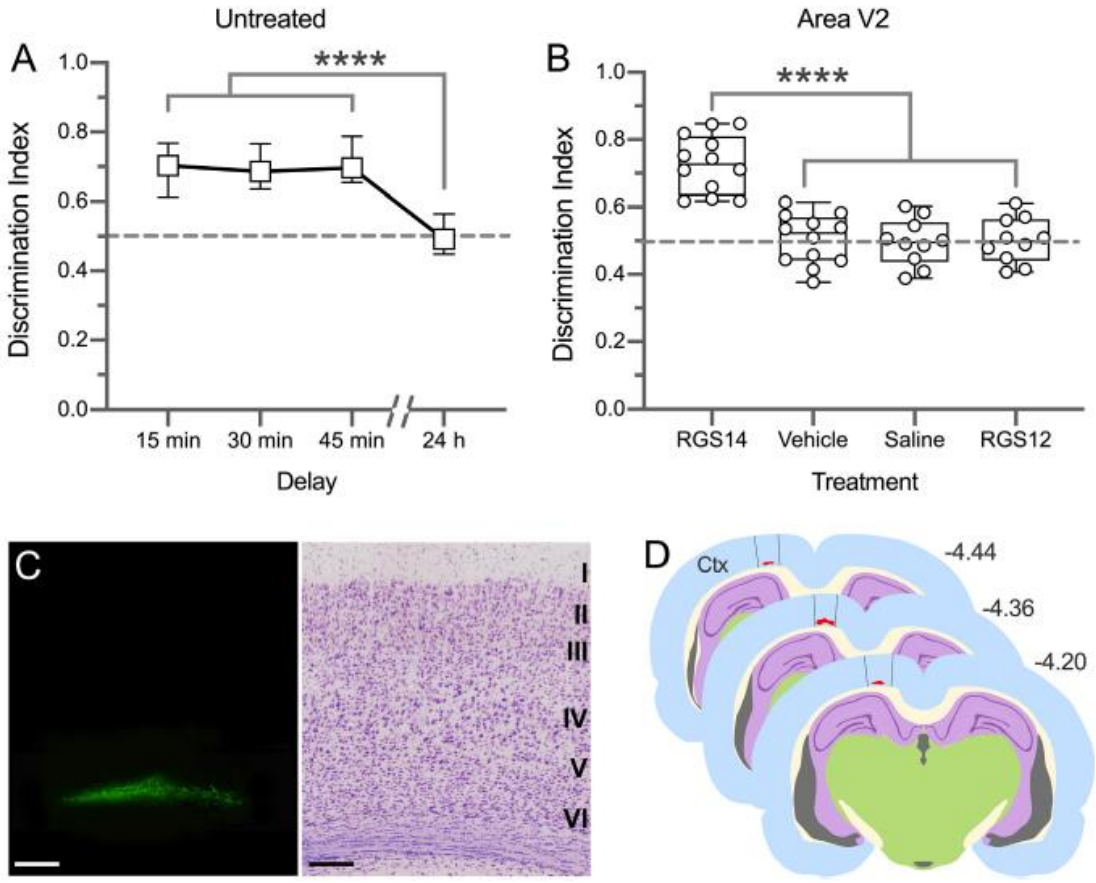

Fig. 1. RGS14$_{414}$ gene treatment induces enhancement in ORM. (A) Three-month-old normal untreated rats were able to retain object information in memory for 15, 30, and 45 min; however, they were unable to retain such information after 24 h. (B) RGS14$_{414}$ gene treatment to the V2 area induced ORM enhancement that could be observed at 24 h, and treatment with vehicle, saline, or RGS12, another gene from the same family, caused no effect. (C) The image on the left shows the immunolabeled RGS14$_{414}$ protein in green, and the image on the right shows a Cresyl Violetstained brain section depicting area V2. Cortical layers (I–VI) are indicated in the right image. Scale bars in both images are 100 mm. (D) A drawing showing the localization of the RGS14$_{414}$ protein (red) obtained after the analysis of serial coronal sections from five RGS14$_{414}$-treated animals and representing the maximum expansion area in all three sections. Area V2 is delimited by thin lines within the cerebral cortex (Ctx). Dotted lines across panels (A), (B) indicate the threshold at which (0.5 DI and below) the animals were unable to retain object information in memory. Each unfilled circle in bars of panels (A), (B) represents the value corresponding to an animal. ****(one-way ANOVA with post hoc Tukey's multiple comparisons test in (A), (B), $p<0.0001$).

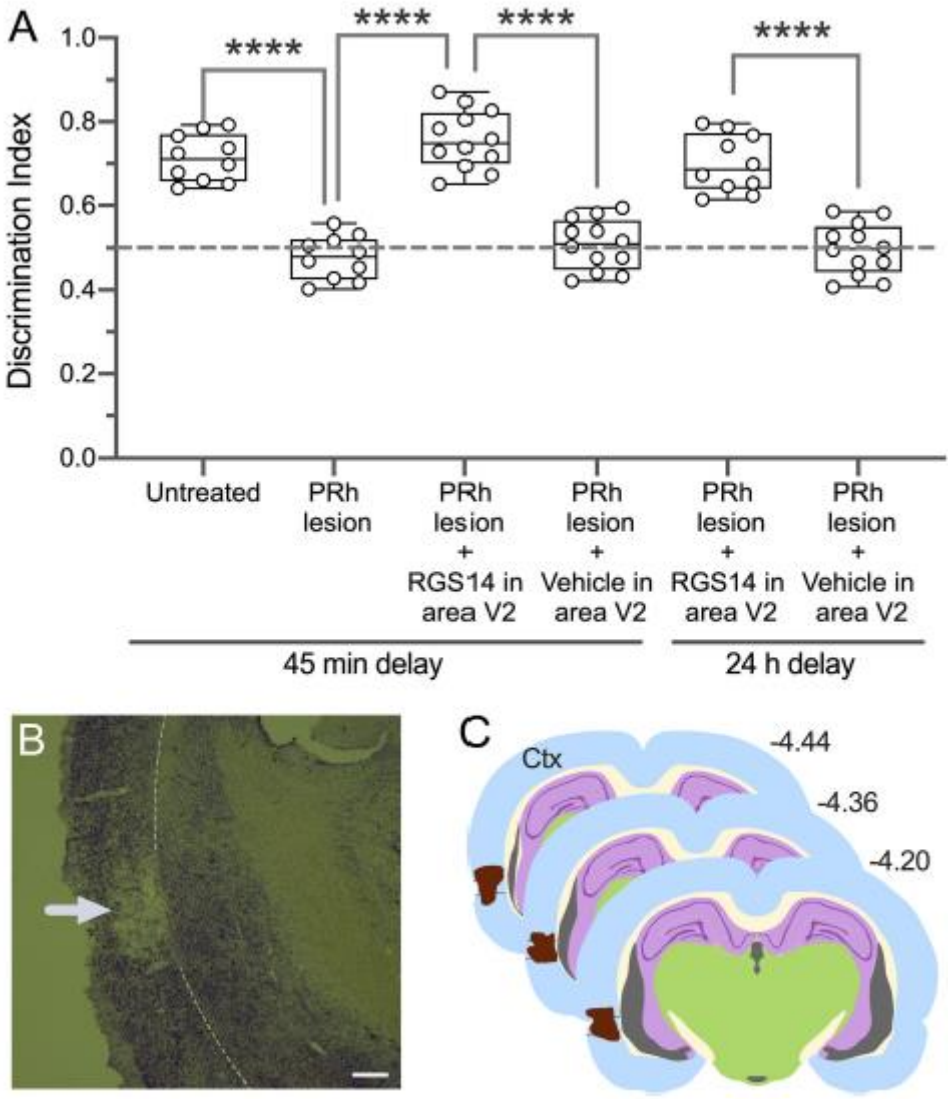

Fig. 2. RGS14414 treatment rescues ORM deficits induced by PRh lesions in rats. (A) The elimination of PRh neurons in rats by immunotoxin Ox7-SAP caused considerable ORM deficit (PRh lesion in 45 min delay) compared with untreated rats, and a treatment of these PRh-lesioned memory-deficient animals with RGS14414 not only led to recovery of ORM of 45 min but also promoted further ORM enhancement. Compared with untreated normal rats (untreated), the RGS14414-treated animals (PRh lesion+RGS14 in area V2) were able to retain object information in memory after a delay of 24 h. However, vehicle treatment did not produce any effect on ORM at delays of both 45 min and 24 h. (B) An example of a Cresyl Violet-stained brain section showing the lesions produced by Ox7-SAP immunotoxin injection to the PRh (arrow). The dotted white line separates the cortical area from the subcortical area. The scale bar=320 mm. (C) A depiction of coronal brain sections obtained after analysis of serial sections from seven PRh-lesioned animals provides a view of the maximum affected area after lesions to the PRh (dark brown). Ctx is the cortex. Dotted lines across panel (A) indicate the threshold at which (0.5 DI and below) the animals were unable to retain object information in memory. Each unfilled

circle in bars of panel (A) represents the value corresponding to an animal. ****(One-way ANOVA with post hoc Tukey's multiple comparisons test, $p<0.0001$).

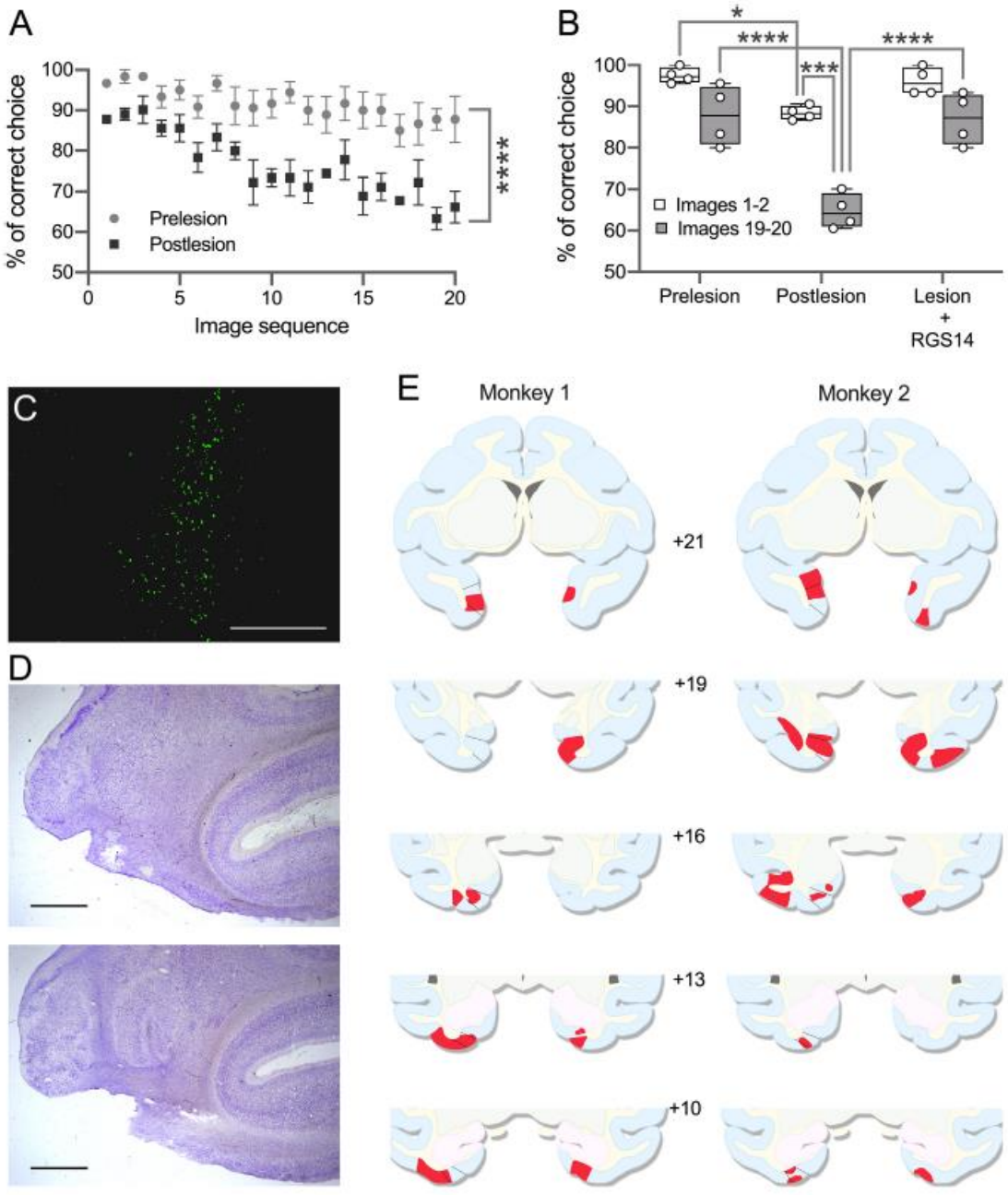

Fig. 3. Reversal of recognition memory deficit induced by PRh lesions in monkeys. (A) Compared with normal monkeys (Prelesion), PRh-lesioned monkeys (Postlesion) subjected to treatment with toxic doses of NMDA showed a continuous but more pronounced decline in their performance on the DNMS test as memory demand was increased by the sequential display of images from 1 to 20. A considerably lower performance was observed in these monkeys at object image positions 19–20. (B) The level of performance at image positions 1–2 was lower in postlesion monkeys (gray bars);

however, this effect was more pronounced at image positions 19–20, for which performance was drastically reduced (black bar). A treatment with the RGS14414 gene to area V2 of these memory-deficient PRh-lesioned monkeys induced complete recovery of memory function (black bar in Lesion+RGS14). (C) A representative image showing the expression of RGS14414 proteins as green fluorescence at the injection site in area V2 of a brain section. Scale bar=400 mm. (D) Two examples of Cresyl Violet-stained brain sections showing the lesions produced by injection of NMDA into the PRh. Scale bars=500 mm. (E) Schematic representation of serial sections indicating the extent of lesions (red) in monkeys 1 and 2. The PRh area is delimited by a thin black line in the left hemisphere of each section. n=2. In panel (B), four unfilled circles correspond to two monkeys where two images (1 and 2 or 19 and 20) of each monkey are represented as two unfilled circles. *(Two-way ANOVA with post hoc Tukey's multiple comparisons test in (B), p=0.0323), ***(two-way ANOVA with post hoc Sidak's multiple comparisons test in (B), p=0.0002), ****(two-tailed unpaired t test in (A) of image positions 19–20, $p<0.0001$; two-way ANOVA with post hoc Tukey's multiple comparisons test in (B), $p<0.0001$).

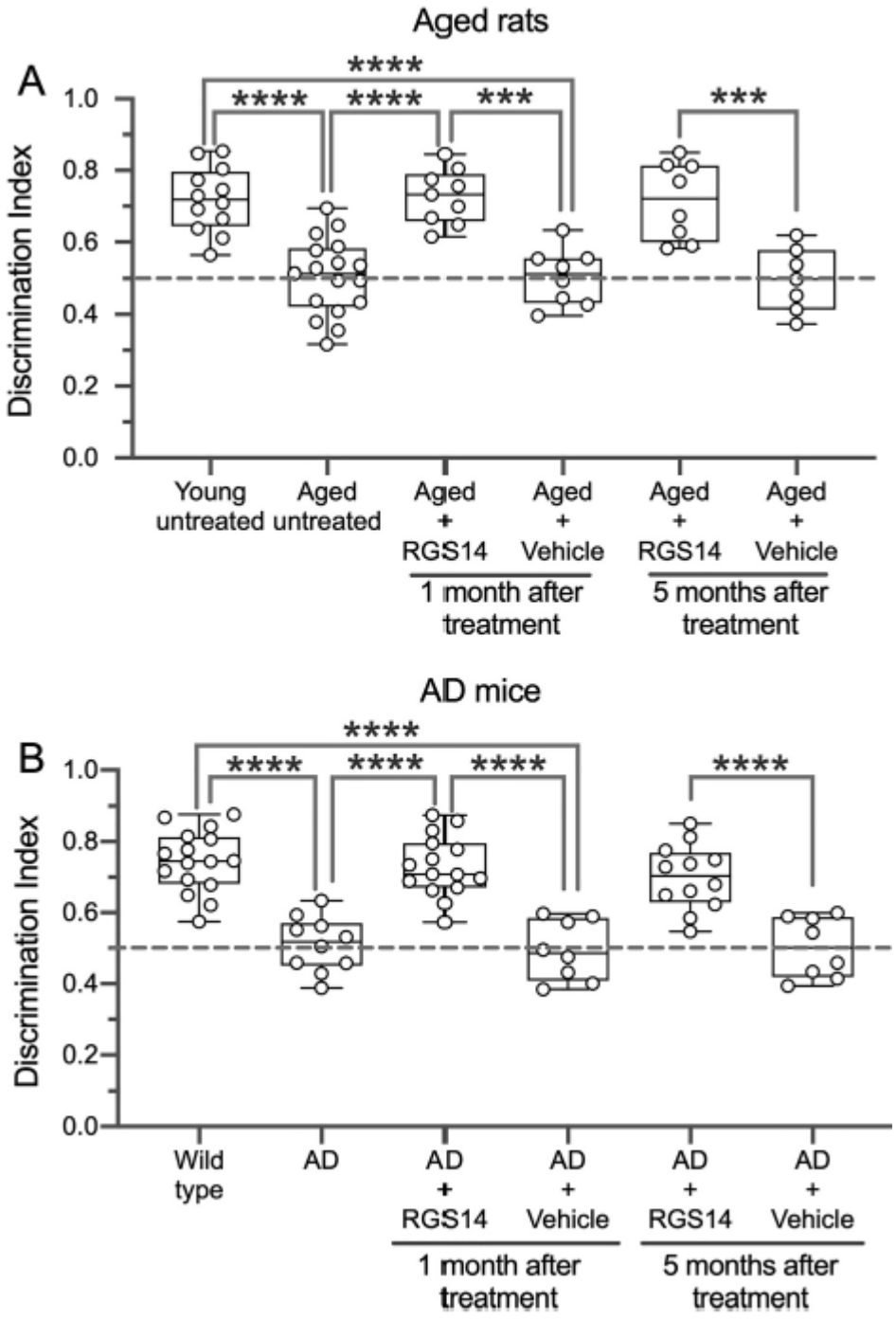

Fig. 4. Recovery of ORM in aging rats and AD mice. (A) When young untreated rats were exposed to an object, they could retain the object information in memory; however, aged untreated rats were unable to retain this information. RGS14414 gene treatment in area V2 of these ORM-deficient aged rats led to full recovery of this recognition memory tested 1 month after the treatment (Aged +RGS14). In contrast, vehicle treatment did not produce any effect (Aged+Vehicle). This recuperated memory level in RGS14-treated animals was maintained even 5 months after the treatment. (B) AD mice showed substantial ORM loss compared with wild-type mice. Akin to aging rats, RGS14414 gene treatment in area V2 of AD mice led to a full recovery of recognition memory tested after 1 month of the treatment (AD+RGS14). In contrast, vehicle treatment did not produce

any effect (AD+Vehicle). The recovered memory level in these RGS14-treated AD mice was maintained even after 5 months of treatment. Dotted lines across panels (A), (B) indicate the threshold at which (0.5 DI and below) the animals were unable to retain object information in memory. Each unfilled circle in bars of panels (A), (B) represents the value corresponding to an animal. *** (One-way ANOVA with post hoc Tukey's multiple comparisons test in (A) p _ 0.0005), **** (one-way ANOVA with Tukey's multiple comparisons test in (A), (B), $p<0.0001$).